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Constitutive expression and inducibility of O^6-methylguanine-DNA methyltransferase and *N*-methylpurine-DNA glycosylase in rat liver cells exhibiting different status of differentiation

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Abstract

We have analyzed the expression of the DNA repair genes O^6-methylguanine-DNA methyltransferase (MGMT) and *N*-methylpurine-DNA glycosylase (MPG) at RNA and protein activity level in primary rat hepatocytes in vitro and various rat hepatoma cell lines exhibiting different status of differentiation. The basal level of MGMT mRNA and activity correlated well with the degree of differentiation, as measured by tyrosine aminotransferase (TAT) mRNA expression. Induction of MGMT mRNA and protein activity by X-ray and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) treatment was most pronounced in the well-differentiated hepatocytes and in various differentiated hepatoma cell lines (up to 6-fold). There was virtually no induction in H5 hepatoma cells which exhibited the lowest degree of differentiation and expressed only low amounts of MGMT. For the other hepatoma cell lines tested, MGMT induction did not clearly correlate with TAT expression. Thus, Fao cells which exhibited a high degree of differentiation responded only very weakly with respect to induction. The results indicate that the basal level of MGMT mRNA expression is dependent on liver-specific regulatory factors, whereas the inducibility is a more complex phenomenon not solely dependent on them. Contrary to MGMT, MPG was constitutively expressed at relatively high amounts in all cell lines tested and no correlation was apparent with the degree of differentiation. MPG activity was significantly induced by mutagen treatment only in H4IIE cells. The tumor promoter phenobarbital induced MGMT, but not MPG mRNA in hepatocytes. The results indicate that MGMT and MPG are not co-regulated. Hepatoma cells with low MGMT level were most sensitive to MNNG-induced cytotoxicity. On the other hand, no correlation was apparent between MPG activity and sensitivity of the cell lines to methylating agents indicating that the MPG level is not predictive for alkylating drug resistance.

Keywords: DNA repair; Gene expression; MGMT; MPG; Hepatocyte; (Rat)

1. Introduction

Damage induced in DNA by monofunctional alkylating agents is repaired in two ways. (i) Alkylations at the O^6-position of guanine are removed by the ubiquitous DNA repair protein, O^6-methylguanine-DNA methyltransferase (MGMT), which transfers the alkyl group to its own active center leading to restoration of guanine in DNA and functional inactivation of the repair protein (for review, see Ref. [1]). (ii) Various other alkylated bases, such as 3-methyladenine and 7-methylguanine, are removed from DNA via the excision repair pathway, the first step of which is catalyzed by the *N*-methylpurine-DNA glycosylase (MPG). This repair enzyme removes *N*-methylpurines by hydrolysis of the *N*-glycosylic bond that links the base to the DNA backbone leaving an apurinic site in DNA which is finally repaired by excision of the apurinic site containing segment and filling in of the resulting gap by repair synthesis and religation [2,3].

In bacteria both repair activities can be induced upon alkylation giving rise to resistance of cells to the mutagenic and killing effect of alkylating agents [4]. For mammalian cells a bulk of data is available showing that the MGMT repair activity and the amount of MGMT mRNA

Abbreviations: MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; MMS, methyl methanesulfonate; PB, phenobarbital; IF, induction factor; MGMT, O^6-methylguanine-DNA methyltransferase; MPG, *N*-methylpurine-DNA glycosylase; TAT, tyrosine aminotransferase; GAPDH, glyceraldehyde phosphodehydrogenase.

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is enhanced upon ionizing radiation, exposure to alkylating agents and other DNA damaging treatments [5–11]. For MPG it is far less clear whether it is inducible on RNA or protein level [9,12].

Induction of MGMT in rat hepatoma cells by MNNG or X-rays gives rise to protection of cells against the mutagenic effect of a challenge dose of MNNG [8]. Induction was also found to protect the cells against the toxic effect of bis-chloroethylnitrosourea [13] and to reduce the frequency of neoplastic transformation in vitro [14]. Furthermore, overexpression of MGMT in transgenic cell lines and in mice exerted protection against methylating agents such as MNNG and *N*-methyl-*N*-nitrosourea to all end points studied so far, including cytogenetic effects, toxicity, gene mutations and tumorigenicity [15–18].

Since MGMT efficiently protects cells against the cytotoxic effect of chemotherapeutic chloroethylating drugs and thus may affect the effectiveness of cancer treatment [19], the constitutive and inducible expression of this repair protein in various organs deserves attention. MGMT was found to be induced most reproducibly and at relatively high levels in rat hepatoma cells in vitro and in the rat liver (reviewed in Refs. [1,12]), raising the question whether MGMT induction is a liver-specific phenomenon dependent on the status of differentiation of the cells. To address this question, we have analyzed the amount of MGMT at the level of RNA and protein activity in various rat hepatoma cell lines that differ with respect to the degree of differentiation and in primary hepatocytes, upon treatment with X-rays or an alkylating agent (MNNG). Furthermore, we were interested to see whether or not MPG is expressed in a similar fashion as MGMT. Our results indicate that the constitutive expression of MGMT mRNA and protein, but not that of MPG, is related to the status of differentiation of rat liver cells. The induction by mutagen treatment of MGMT mRNA and protein was most marked in the differentiated cell lines and in primary hepatocytes, but there was no clear-cut relation to the status of differentiation, indicating that complex regulatory mechanisms are involved in MGMT induction.

2. Materials and methods

2.1. Cell culture

The rat hepatoma cell lines H5, H4IIEC3/T, H4IIEC3/G$^-$ and Fao were obtained from F.J. Wiebel (GSF, Munich, Germany) and have been described previously [20]. The H4IIE cell line was delivered from K.-L. Lee (Oak Ridge National Laboratories, USA). These cells have been used in previously reported studies [8]. All cell lines, except H4IIE, were cultivated in F12/Dulbecco's medium (1:1) containing 10% fetal calf serum (Gibco). The H4IIE cells were cultivated in alpha modified Eagle's medium containing 5% fetal calf serum and antibiotics (50 μg/ml gentamycin).

2.2. Animals

Male Sprague-Dawley CD rats weighing 150 g were obtained from Charles River (Germany). The animals were acclimatised for a period of 1 week in a 12 h dark-light-rhythm and fed ad libidum. The animals were starved 24 h before liver perfusion.

2.3. Liver perfusion and culture of hepatocytes

Liver perfusion was performed by a two-step collagenase perfusion protocol as described [21]. In brief, the isolated liver was first perfused by a calcium and magnesium free medium to disrupt the cell–cell connections and then with collagenase solution to disrupt the extracellular matrix. For protection, the solution contained trypsin inhibitor and DNase. The cells were then washed out of the liver. To enrich vital hepatocytes, the cells were subjected to Percoll (Pharmacia) density gradient centrifugation. The vital hepatocytes gathered at the bottom of the gradient, whereas non-hepatocytes formed a layer at the top of the Percoll solution. The viability of the hepatocytes was $> 90\%$ which was measured by trypan blue exclusion. For aggregation to spheroids, $2.5 \cdot 10^6$ hepatocytes were cultivated using Weymouth medium (Gibco) containing 10% CPSR-1 (Sigma) for 48 h on uncoated 10 cm-dishes (10 ml each), with a medium change 24 h after seeding. 48 h after cultivation, hepatocytes which had aggregated to spheroids were transferred into Spinner-cell culture bottles for further cultivation [22].

2.4. Drugs and cell treatment

MNNG and phenobarbital were obtained from Sigma. MNNG was dissolved in a small amount (approx. 200 μl) of dimethylsulphoxide, diluted in distilled water and stored in aliquots at -80°C. MNNG from the stock solution (10 mM) was added to the medium of exponentially growing cells or to the hepatocyte spheroid cultures (final concentration of 15 μM). Phenobarbital was dissolved prior to use in 100 mM NaOH and diluted in 30 ml medium which was then neutralized by 100 mM HCl. The final concentration of the drug was 1.5 mM. X-ray irradiation was performed with exponentially growing hepatoma cells that were attached to the culture dishes. For irradiation of the hepatocytes, they were collected in 50 ml plastic tubes and then the medium was aspirated to give a final volume of 10 ml. Immediately after irradiation fresh medium was added to the cells. They were harvested 24 h and 48 h after irradiation for RNA and protein assays, respectively.

2.5. Plasmids

pAPDG 10 containing the rat MPG cDNA and pcDNAII containing the rat MGMT cDNA were kind gifts from Drs. F. Laval and T.R. O'Conner (Paris). For hy-

bridization, the MPG and MGMT cDNA inserts were excised from the plasmids and purified by gel electrophoresis.

2.6. RNA extraction and Northern blot hybridization

Total RNA was prepared by the guanidinium-isothiocyanate-phenol-chloroform method [23]. 20–50 μg of total RNA was denatured in formaldehyde buffer and separated on a 1% agarose gel without the inclusion of formaldehyde. The RNA was visualized by ethidium bromide and transferred onto a Gene Screene Plus (NEN, DuPont) or Hybond N^+ (Amersham) nylon membrane in 20 × SSC solution [11].

2.7. Hybridization

cDNA probes of MGMT and MPG were labeled using the random priming procedure according to the manufacturer's protocol (Stratagene) yielding a specific activity of $5 \cdot 10^8$–10^9 cpm/μg. Hybridization was performed at 60°C in 1 M NaCl, 1% SDS, 10% dextran sulfate containing 100 μg/ml denaturated salmon sperm DNA. For washing, decreasing concentrations (1 × and 0.5 ×) of SSC and 0.5% SDS were used at 60°C. The wet filters were wrapped in sealable plastic bags and exposed to X-ray films. For rehybridization, the filters were stripped by incubating in 1% SDS at 80°C for 0.5 h. The filters were washed in 2 × SSC and resealed wet. The amounts of mRNA hybridized onto the filter were quantified by densitometric scanning of the films. Differences in loading were normalized by the amount of GAPDH mRNA in the same sample. Induction factors were calculated in relation to GAPDH mRNA, which were not found to be affected by the treatments applied [11].

2.8. Protein isolation

Total protein was prepared from exponentially growing cells. During the preparation the protein extract was kept on ice and all solutions were cooled prior to use. Cells were washed twice with PBS and collected by centrifugation (1200 rpm, 4 min). Cells were disrupted by sonication in 70 mM Tris (pH 8), 1 mM EDTA, 5% glycerol and the cell debris removed by centrifugation. The supernatant was aliquoted and immediately frozen in liquid nitrogen. The protein content in the supernatant was determined by the Bradford dye-binding assay (Bio-Rad).

2.9. MGMT activity

MGMT activity was determined by the transfer of ^{3}H-labeled O^6-methylguanine from a DNA template to protein. The DNA was from salmon sperm (Sigma) and labeled with [^{3}H]methyl-*N*-nitrosourea (16 Ci/mmol, Amersham) as described [15]. In a standard reaction 200 μg of cell extract protein was incubated at 37°C with ^{3}H-labelled DNA (120000 cpm) in 70 mM Hepes, 1 mM DTT, 5 mM EDTA, pH 7.8. After 30 min the reaction was stopped by the addition of 11 μl trichloroacetic acid and the DNA hydrolyzed by incubating for 15 min at 100°C. The precipitated protein was collected by centrifugation and washed three times with 5% TCA. Finally the protein was solubilized in 0.1 N NaOH by heating and the radioactivity determined in a liquid scintillation counter. The MGMT activity was calculated as fmol of [^{3}H]methyl transferred to TCA-insoluble material per mg total cell extract protein. Since cells of the lines studied differ considerably in size, the repair activity was recalculated for a given number of cells. For this purpose the protein content of the cells was determined. It amounted to 80 μg/10^6 H5 cells, 210 μg/10^6 H4IIE cells, 180 μg/10^6 H4IIEC3/T cells, 300 μg/10^6 H4IIEC3/G$^-$ cells, 240 μg/10^6 Fao cells, and 3430 μg/10^6 hepatocytes. MGMT activity was expressed as fmol/10^6 cells. As a control included in each assay served boiled extracts of Mex$^-$ HeLa MR cells or bovine serum albumine. The measured control level of radioactivity (approx. 120 cpm per 200 μg protein) was subtracted from the activities detected in the liver cell extracts.

2.10. MPG activity

MPG activity was determined by the release of ^{3}H-labelled 3-methyladenine and 7-methylguanine from alkylated DNA template. The DNA substrate was salmon sperm DNA that was treated with [^{3}H]dimethylsulfate (DuPont de Nemours, Bad Homburg; 1.1 Ci/mmol; 5 mCi for 20 mg DNA in 10 ml of 10 mM Tris, 1 mM EDTA, pH 7.5 for 12 h at room temperature; specific activity of the DNA: 0.541 μCi/mg). Spontaneously hydrolyzed methylated bases were removed from the template DNA prior to use by column chromatography through Sephadex G-75. In a standard reaction, 300 μg of cell extract protein was incubated at 37°C with ^{3}H-labelled DNA (60000 cpm) in 100 mM KCl, 50 mM Hepes (pH 6.7) and 5 mM DTT. After 1 h, the reaction was stopped by cooling on ice. The DNA substrate was precipitated by the addition of 50 μg salmon sperm DNA, 0.1 vol. 3 M sodium acetate and 2 vol. ethanol and collected by centrifugation. The supernatant containing the released bases was dried in a speedvac and the pellet was dissolved in 40 μl FPLC-running buffer (0.4 M $NH_4H_2PO_4$, 5% methanol). The ^{3}H-labelled methylated bases were separated by reverse phase FPLC through a PepRPC HR5/5 column (Pharmacia) in FPLC running buffer. The flow rate was 0.5 ml per min. Prior to chromatography, 10 μl of a mixture of methylated purines (3-methyladenine, 1-methyladenine, 1-methylguanine, 7-methylguanine; 1 mg/ml each) was added. The elution of the purines was followed up by UV spectrophotometry. The fractions (0.5 ml each) containing the methylated bases were measured for their ^{3}H content. MPG

activity was determined as fmol of methylated bases (3-methyladenine and 7-methylguanine) that were released per mg of total protein per hour and expressed, taking into account the different protein content of the cell types (see MGMT assay), as fmol per 10^6 cells per hour.

2.11. Survival assays

300 to 600 cells from exponentially growing cultures were seeded per 6-cm dishes and treated 6 h later with MNNG or MMS by adding the chemicals from a stock solution directly to the dishes. 1 h after adding the mutagen, the medium was removed and new medium was added. Colonies were fixed 7–14 days after seeding, depending on the growth of the cells, with methanol and stained with crystal violet. Survival frequency was expressed as the number of colonies on the treated plates divided by the number of colonies that appeared on the control dishes. The plating efficiency for H5 cells was approx. 50%, for H4IIEC3/T cells approx. 40%, for H4IIEC3/G$^-$ cells approx. 25% and for Fao cells approx. 65%.

3. Results

3.1. Basal level of expression of MGMT and MPG in rat hepatoma cell lines and hepatocytes

The rat hepatoma cell lines we have chosen for our studies (H5, H4IIE, H4IIEC3/T, H4IIEC3/G$^-$, Fao) have been reported to differ significantly in their status of differentiation, as measured by the expression of aldrin epoxidase which is a liver-specific cytochrome *P*-450 function, and other markers [20,24–26]. Furthermore, the rat hepatocytes freshly isolated and maintained in culture as spheroids were previously shown to maintain a highly differentiated status in that they express cytochrome *P*-450 mono-oxygenase over the entire cultivation period assayed, i.e., up to 1 week [22]. In our experiments, we have used, as an indicator of liver-specific differentiation, the expression of TAT which is considered to be a liver-specific marker [27].

As shown in Fig. 1 (and Table 1 for quantification) the H5 line does not significantly express TAT mRNA. Also the aldrin epoxidase activity was not detectable in this cell line (Table 1). TAT is expressed, however, in increasing amounts in the lines H4IIEC3/T, H4IIE, H4IIEC3/G$^-$ and Fao. This agrees reasonably well with previous studies based on other liver-specific markers [20] and allows the conclusion that these cell lines are liver-specific 'differentiated' (Fao, H4IIEC3/G$^-$, H4IIE) or 'dedifferentiated' (H5, H4IIEC3/T). The highest level of TAT was found in the hepatocyte spheroid culture, which is in line with the high level of cytochrome *P*-450 mono-oxygenase expressed under these culture conditions [22] indicating that the freshly isolated hepatocytes maintained their liver-specific status of differentiation for at least 1 week during which they were cultivated as spheroids in vitro.

The basal activity of MGMT and MPG in the hepatoma cell lines and hepatocytes is shown in Table 1. H5 expressed low, but significant amount of MGMT (4 fmol/10^6 cells or 52 ± 19 fmol/mg protein). An intermediary level of expression was found in H4IIEC3/T and H4IIE, and a

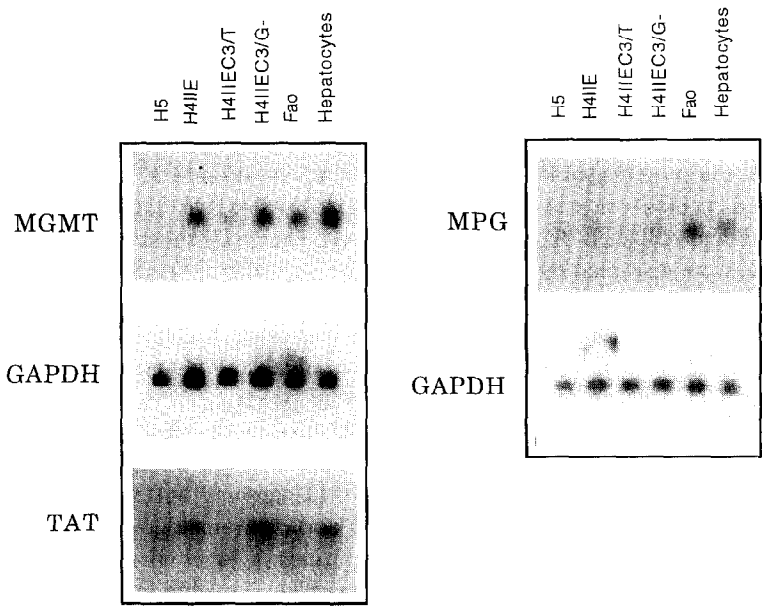

Fig. 1. Expression of MGMT, MPG, and TAT mRNA in various untreated rat hepatoma cell lines and rat hepatocytes. 10 μg of total RNA was applied per slot. Northern blots for MGMT and MPG are from separate experiments (no reprobing). The membranes were re-hybridized with GAPDH in order to correct for the amount of mRNA.

Table 1
MGMT and MPG activity in rat hepatoma cell lines and primary rat hepatocytes not treated (basal level) or treated with MNNG or X-rays

Cell type	MGMT (fmol/10^6 cells)					MPG (fmol [3MeA + 7MeG]/10^6 cells × h)					TAT [a]	Aldrin epoxidase [b]
	basal level	MNNG		X-ray		basal level	MNNG		X-ray			
			I.F.		I.F.			I.F.		I.F.		
H5	4 ± 2	0.4	0.1	4	0.9	65 ± 3	60	0.9	90	1.3	b.d.	b.d.
H4IIE	113 ± 23	196	1.7	176	1.6	74 ± 14	251	3.4	224	3.0	0.116	
H4IIEC3/T	70 ± 6	117	1.6	124	1.8	68 ± 22	110	1.6	100	1.3	0.05	0.8
H4IIEC3/G$^-$	204 ± 47	183	0.9	199	1.0	178 ± 39	279	1.6	236	1.3	0.229	18.1
Fao	159 ± 6	210	1.3	225	1.4	169 ± 52	228	1.3	111	0.7	0.364	29
Hepatocytes	192 ± 164	1015	5.3	518	2.7	782 ± 324	1252	1.6	1427	1.8	1.379	

Freshly isolated hepatocytes were grown as spheroids. Cells were harvested for extract preparation 48 h after the addition of MNNG (15 μM) or X-ray irradiation (2 Gy). Each value represents the average of three independent determination. b.d., below level of detection; I.F., induction factor. For comparison, the level of TAT mRNA expression and aldrin epoxidase enzyme activity is shown.

[a] TAT expression is given in arbitrary units calculated from densitometric measurement of the amount of TAT mRNA and GAPDH mRNA (Fig. 1), and is expressed in relation to GAPDH mRNA.

[b] Data for aldrin epoxidase activity (pmol/min per mg) are from Hesse et al. [20].

high level in Fao, H4IIEC3/G$^-$ and hepatocytes. Thus, all cell types included in this study are phenotypically Mex$^+$. The level of MPG activity present per cell did not correlate with MGMT activity. Thus, H5 cells expressed nearly as much MPG as H4IIE. An exceptional high amount of MPG activity was found in hepatocytes.

In order to see whether the expression of MGMT and MPG is related to the extent of liver-specific differentiation, RNA was isolated from cell lines and from hepatocytes, blotted and probed against MGMT, MPG, GAPDH (which served as an internal standard for the amount of RNA), and TAT. As shown in Fig. 1, H5 cells did not exhibit detectable amounts of MGMT mRNA. The constitutive MGMT mRNA level increased in the order H4IIEC3/T, H4IIE, H4IIEC3/G$^-$, Fao and primary hepatocytes. Overall, both MGMT and MPG mRNA paralleled the corresponding repair activity. An exception is provided by the hepatocytes whose high MPG activity was not reflected by a high amount of mRNA (in relation to GAPDH), as compared to the other cell types.

The expression of MGMT and MPG mRNA as a function of TAT is shown in Fig. 2. It is obvious that the amount of MGMT mRNA increases with TAT expression. This was not observed for MPG mRNA. Thus the constitutive level of MGMT, but not MPG mRNA appears to correlate with the status of differentiation. The data also indicate that the basal level of MPG and MGMT is not coordinately regulated in liver-derived cell lines and hepatocytes.

3.2. Inducibility of MGMT

The effect of X-ray and MNNG treatment on the level of MGMT mRNA in rat hepatoma cell lines and hepatocytes is shown in Fig. 3. A significant induction of MGMT mRNA was observed for H4IIEC3/T cells (up to 6.5-fold) and for the lines H4IIE and H4IIEC3/G$^-$ (2- to 6-fold). Only a very weak response was found in Fao and none at all in H5 cells. Hepatocytes responded to treatment with X-rays with relatively strong induction of MGMT mRNA (5.3-fold) which is comparable to the level found in H4IIE cells. The level of induction of MGMT mRNA after X-ray and MNNG treatment in dependence of TAT expression is shown in Fig. 4. No correlation was found between MGMT mRNA induction and TAT expression.

The induction of MGMT mRNA was accompanied by an increase in MGMT activity (Table 1). The strongest induction of MGMT repair activity was observed in hepatocytes (up to 5-fold with 15 μM of MNNG). It should be noted that, upon MNNG treatment, MGMT is depleted to some extent due to repair of the induced O^6-methylguanine and possibly also by direct methylation. This might explain

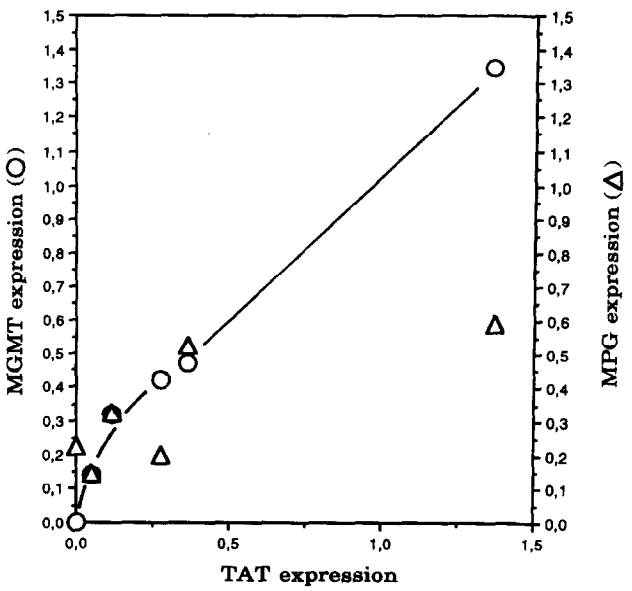

Fig. 2. Dependence of MGMT (○) and MPG (△) mRNA expression on the level of TAT mRNA. The mRNA expression is given in arbitrary units, which are the quotient of MGMT/GAPDH, MPG/GAPDH and TAT/GAPDH, respectively. Data were obtained by densitometric scanning of the autoradiograms shown in Fig. 1.

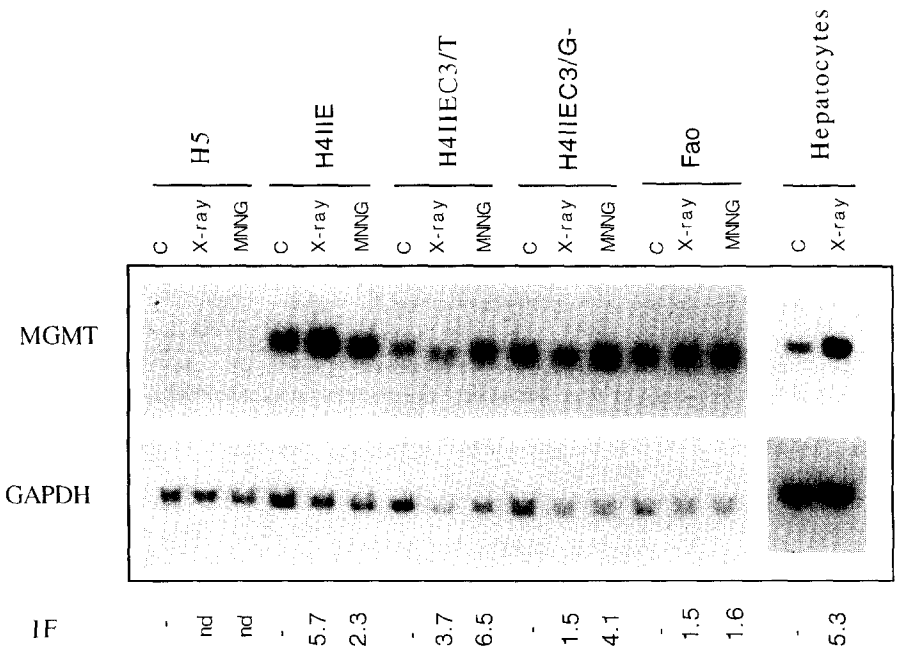

Fig. 3. Northern blot analysis of MGMT mRNA of rat hepatoma cell lines and hepatocytes treated with MNNG or X-rays. RNA was extracted 24 h after irradiation (2 Gy) or the addition of MNNG (15 μM) to the medium. The membrane was reprobed with GAPDH. The induction factor (IF) represents the relative amount of MGMT mRNA (MGMT/GAPDH) after treatment, in relation to the corresponding untreated control (C) that was included in each experiment. nd, not detectable.

why the MNNG-treated H5 cells displayed significantly lower MGMT activity than the control.

3.3. Inducibility of MPG

The MPG mRNA levels 24 h after mutagen treatment in exposed and non-exposed liver cell lines and hepatocytes are shown in Fig. 5. There was no significant induction of MPG mRNA. The MPG activity was measured 48 h after mutagen treatment and was found to be significantly enhanced in H4IIE cells (3- and 3.4-fold for X-rays and MNNG, respectively). No or only very slight increase above the basal level was observed in the other cell lines. Rat hepatocytes showed only a weak (up to 1.8-fold) increase in MPG activity after X-ray and MNNG treatment (Table 1). Fig. 6 shows, for comparison, the magnitude of induction of MGMT and MPG activity in the liver cell lines and hepatocytes. It appears that MGMT and MPG activity is not coinduced upon treatment with X-rays and MNNG.

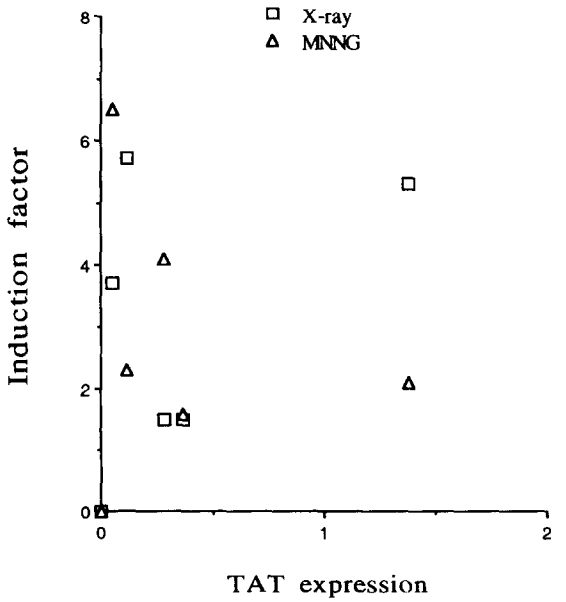

Fig. 4. Relation between MGMT and TAT expression in liver cells exposed to MNNG or X-rays. MGMT and TAT mRNA levels are given as arbitrary units (see Table 1 and Fig. 2). Data are from densitometric measurements of the autoradiograms shown in Fig. 3 and, for MNNG treated hepatocytes, Fig. 7.

3.4. Effect of phenobarbital

Phenobarbital is a potent tumor promoter in rat liver [28]. To see whether it is effective in inducing MGMT and MPG, rat hepatocytes were treated with the agent and the amount of MGMT and MPG mRNA was determined. In the same set of experiments treatment with MNNG was included. As shown in Fig. 7, both MNNG and phenobarbital induced MGMT, but not MPG mRNA.

3.5. Sensitivity of rat hepatoma cell lines to alkylating agents

To analyze the cellular sensitivity to alkylating agents in relation to the expression of MGMT and MPG, the survival of rat hepatoma cells was determined after exposure to MNNG and MMS (Fig. 8). H5 cells, which dis-

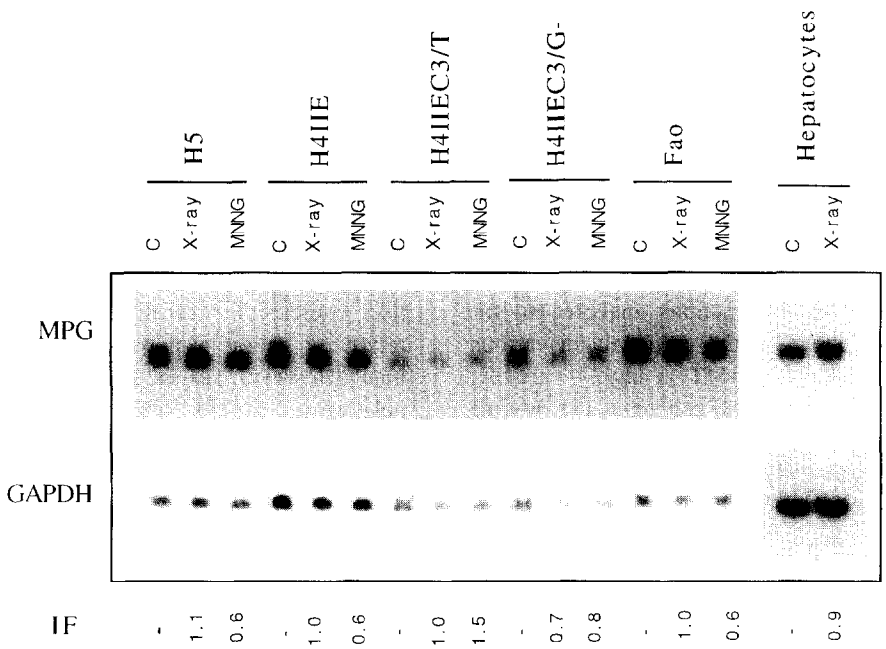

Fig. 5. Northern blot analysis of MPG mRNA of rat hepatoma cell lines and hepatocytes treated with MNNG or X-rays. RNA was extracted 24 h after mutagen treatment (15 μM for MNNG, 2 Gy for X-rays). The membrane was re-hybridized with GAPDH. Induction factors (IF) were determined as described in Fig. 3.

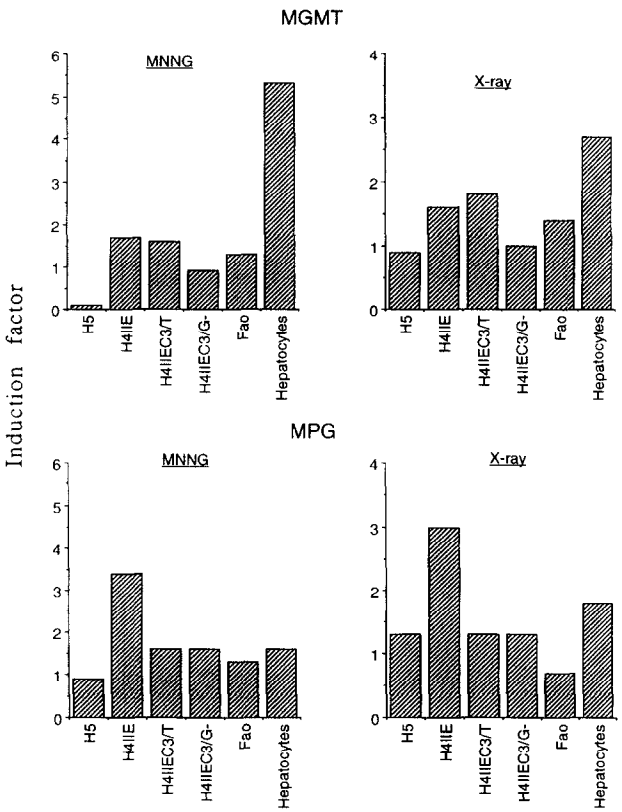

Fig. 6. Comparison of induction of MGMT and MPG protein activity by X-ray (2 Gy) or MNNG (15 μM) treatment. Induction factors are from data shown in Table 1, and are related to the basal activity of each cell type.

played the lowest level of MGMT, were clearly more sensitive to MNNG than the other lines. These cells, however, were not more sensitive to MMS. An exceptional behavior showed Fao cells which were more resistant than the other cell lines to MMS. In the survival studies CHO-9 cells were included for comparison. These cells do not express detectable amounts of MGMT and are comparable to H4 with respect to MPG activity (unpublished data).

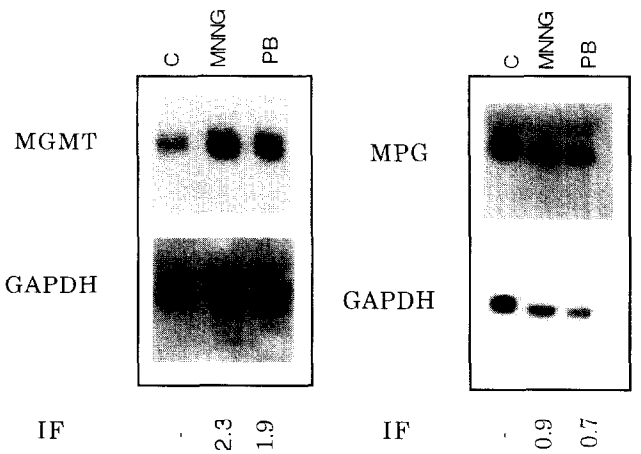

Fig. 7. Northern blot analysis of MGMT and MPG mRNA of rat hepatocytes treated with phenobarbital (1.5 mM) or MNNG (15 μM). C, untreated control. The RNA was extracted 24 h after drug exposure. The blots for MGMT and MPG are from separate experiments. They were reprobed with GAPDH.

CHO-9 cells are clearly hypersensitive to MNNG, but not to MMS. Thus, it appears that the sensitivity to MNNG is related to MGMT expression and that the MPG activity seems not to affect the resistance to MNNG and MMS.

4. Discussion

Whereas most DNA repair functions analyzed so far appear to be expressed more or less uniformly in various cell types, the expression of MGMT is highly variable, depending on the species, tissue and cell type, and in vitro growth characteristics (for review see Refs. [1] and [12]). MGMT is present at relatively high amount in the liver which is due to the high level of expression in hepatocytes. In non-parenchymal liver cells, only low amounts of MGMT have been found [29]. In addition to the high constitutive level, induction of MGMT mRNA and protein was most consistently observed and at comparatively high level in rat liver and rat hepatoma cells in vitro (for review see Ref. [12]). Thus, treatment of rat hepatoma cells with DNA damaging agents induced MGMT mRNA 2–5-fold [8–11]. For rat liver, an induction was reported of up to 10-fold in MGMT mRNA upon treatment of animals with 2-acetylaminofluorene [30] and up to 20-fold in MGMT activity upon exposure to ionizing radiation [31]. It should be noted that induction is not restricted to rat liver cells. It was also found in mouse C3H10T1/2 cells [32] and in various mouse tissues [33].

The high constitutive expression of MGMT and its relatively strong inducibility in rat liver cells raised the question whether liver-specific transcription factors are involved in regulation of the MGMT gene. If liver-specific transcription factors are involved, it would be expected that MGMT expression is related to the expression of the genes that are regulated in a liver-specific fashion. Here we have shown that the basal level of expression of MGMT mRNA and activity correlates with the amount of TAT mRNA and thus is related to the status of differentiation of liver cells. These results confirm and extend a previous report based on measurement of MGMT activity in rat hepatoma cell lines [20]. One could speculate that the induction of MGMT in rats is also related to the liver-specific status of differentiation since MGMT is most efficiently induced in liver cells. In line with this would be that H5 cells exhibiting the lowest degree of differentiation did not show detectable MGMT mRNA and protein induction whereas the well differentiated hepatocytes elicited a relatively strong response after X-ray and MNNG treatment. However, for the other hepatoma cell lines, we did not find a clear-cut correlation between induction and the degree of differentiation. Thus, Fao cells which are highly differentiated (in terms of TAT and aldrin epoxidase expression) and displayed a high basal level of MGMT mRNA showed only very weak MGMT mRNA induction. This indicates that a high degree of differentiation (does not necessarily enable the cells to respond strongly with MGMT induction. Induction depends on the presence of an inducing signal (probably DNA damage, Ref. [11]). We cannot exclude the possibility that Fao cells are more efficient than the other cell types in eliminating the inducing signal and thus they could also be potentially responsive to induction. It is also obvious from the results obtained that a low basal level of MGMT mRNA and protein is not decisive for its induction, and that a high basal level, as observed in hepatocytes and various hepatoma cell lines, does not prevent the gene to be further activated by exposure to mutagens. The increase of MGMT mRNA upon mutagen treatment has been concluded to be due to transcriptional activation of the gene since the RNA synthesis inhibitor actinomycin D abolished MGMT mRNA accumulation [8] and since MGMT induction was also observed in nuclear run-on assays [31].

We have found, in spheroid cultures of primary rat hepatoma cells, a maximum of induction of MGMT (on RNA level) by approx. 6-fold. There are several possible reasons why we did not observe a similar high level of

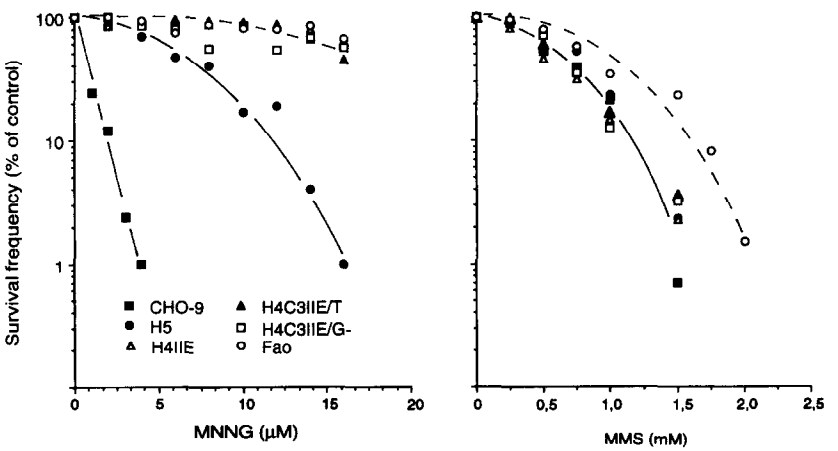

Fig. 8. Survival of rat hepatoma cell lines upon treatment with MNNG or MMS. For comparison the Mex$^-$ Chinese hamster cell line CHO-9 was included in this study. Each point represents the mean value of three independent experiments.

induction in hepatocytes in vitro, as compared to in vivo experiments [31]. 1) The hepatocytes became dedifferentiated immediately after their isolation and during their maintenance in culture (for up to 1 week). Thus, cytochrom *P*-450 and aldrin epoxidase activity was shown to decrease by about 40–60% in our hepatocyte spheroids at the end of the cultivation period [22]. 2) Maximum of induction in the liver was found with 15 Gy of irradiation [31]. We used a relatively low dose of 2 Gy since we were interested in studying the effect of the mutagen under conditions that allowed the cells to survive. Increasing the dose of both X-rays and MNNG enhanced induction but also elicited strong toxic effects (data not shown). 3) The hepatocytes were derived from male rats. We cannot exclude a gender effect as previously reported [31]. Nevertheless, the relatively high induction level of MGMT in primary hepatocytes grown as spheroids make them a suitable experimental system for studying the regulation of MGMT in relation to liver-specific functions.

All DNA damaging treatments studied so far, including the electroporation of restriction enzymes, gave rise to induction of MGMT mRNA and protein which led to the conclusion that DNA breaks are the ultimate signal for eliciting the response [7,11]. This, however, does not exclude the possibility that physiological signals can also act as inducers. In this context it should be noted that the liver-specific tumor promoter phenobarbital induced MGMT mRNA in hepatocytes. Phenobarbital is not a DNA damaging agent. It induces alterations in gene expression in the liver [34] apparently involving MGMT as well.

Previously it has been reported that the MPG repair activity and MPG mRNA are enhanced after mutagen exposure of H4 cells [9,12,35]. It should be noted, however, that it is not clear whether MPG is inducible on the transcriptional level or whether post-transcriptional mechanisms, such as mRNA and protein stabilization, are involved. Here we have shown that 24 h after mutagen treatment, MGMT mRNA was induced in differentiated hepatoma cell lines and hepatocytes, but there was no significant induction of MPG mRNA. The MPG activity was assayed 48 h after mutagen exposure and was found to be enhanced up to 3.4-fold in only one cell line (H4IIE). The results are in line with our previous observation that MPG induction in H4IIE cells is delayed with the maximum response occurring 48 h after mutagen exposure (Hartenstein and Kaina, unpublished data). Induction of MPG activity was related neither to the basal level of MPG mRNA and enzyme activity nor to the status of differentiation of liver cells. Thus the hepatocyte spheroid cultures exhibited only a very weak response. This again indicates that MPG and MGMT are not coregulated. Lack of coregulation is supported by the finding that MGMT, but not MPG is induced by phenobarbital, and that MPG is expressed quite uniformly in Mex^- and Mex^+ human and rodent cell lines (unpublished data).

A deeper insight into the molecular mechanism of MGMT and MPG regulation requires cloning of the promoter sequences of the genes. A human MGMT promoter fragment was isolated and shown to contain various regulatory elements including a glucocorticoid responsive element [36] indicating that humoral control factors are possibly involved in MGMT regulation. It should be noted, however, that MGMT was not inducible by mutagen treatment in human fibroblasts and the human hepatoma cell line HepG2 [8]. Whether MGMT can be induced in well differentiated human hepatocytes is still an open question.

Whereas MGMT exerts protection, the role of MPG for determining a given level of alkylating drug resistance is still unclear. Data obtained from transgenic cell lines indicate that both O^6-methylguanine and *N*-methylpurines are cytotoxic, depending on the agent used for alkylation [37]. On the other hand, increased expression of MPG did not enhance resistance of Chinese hamster cells to alkylating drugs [38]. Comparing the sensitivity of various rat hepatoma cell lines to alkylating agents, we found H5 exhibiting the lowest basal level of MGMT and no MGMT induction, to be most sensitive to MNNG. H5 cells, however, were not sensitive to MMS indicating that MNNG-induced cytotoxicity is brought about mainly by O^6-methylguanine whereas for MMS, other lesions are decisive. This conclusion is in agreement with results obtained from transgenic cell lines [37]. Interestingly, Fao cells proved to be significantly more resistant to MMS although the level of MPG and MGMT was comparable to other lines, e.g., H4IIEC3/G^- cells. Possibly an unknown mechanism independent of MGMT and MPG is operating in these cells that protects them against MMS-induced toxicity. Overall, for MPG, no correlation was found between its expression and the cell killing effect of MNNG and MMS indicating that the MPG level is not a determinant of sensitivity of cells to the cytotoxic effect of alkylating drugs.

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